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A Comparison of Age Structures in Two Populations of a Pond Frog *Rana nigromaculata* (Amphibia: Anura)

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ABSTRACT—Using skeletochronology, we compared age structure in the breeding season in two populations (Katata, Shiga Pref. and Shizuhara, Kyoto Pref.) of a pond frog, *Rana nigromaculata* from central Japan and elucidated interpopulation variation in some life history traits. Newly metamorphosed froglets were larger in SVL, heavier in body mass, and fatter in Katata than in Shizuhara. In both populations, the age at first reproduction was 2 yrs for males and 3 yrs for females. Longevity was estimated to be at least 5 and 6 yrs at Katata and Shizuhara, respectively, for both sexes. Females were on an average significantly older than males in both populations. The modal age in breeding males was 2 yrs in Katata but 4 yrs in Shizuhara, and Katata males (mean=3.27 yr) tended to be younger than Shizuhara males (mean=3.42 yr), although the difference was not significant. Similarly, females did not differ in the mean age in the two populations (4.1 yrs in both populations). In SVL, females were significantly larger than males in both populations. In males, SVL was larger in Shizuhara than in Katata, but females did not differ in SVL. Shizuhara males grew faster than Katata males, but females did not differ in growth rate in both populations. Interpopulation differences in age and size properties of *R. nigromaculata* seem to be related to degree of interspecific competition and food consumption. We briefly discussed use of skeletochronology in relation to amphibian declining problems.

INTRODUCTION

The technique of skeletochronology is recently becoming more and more popular in accurately determining ages in amphibians (e.g., Kusano *et al.*, 1995a, b; Misawa and Matsui, 1999). The accurately assessed individual age permits us greater understanding of life history traits in this group of animals.

Rana nigromaculata Hallowell, 1861 occurs in plains and hillsides around rice fields of east Asia including Japan (Maeda and Matsui, 1999; Hirai and Matsui, 1999). This species occurs syntopically with its relative, *R. porosa brevipoda* in some parts of Japan, and where they co-occur, the two species sometimes show a strong competition in breeding activities (Shimoyama, 1996; 1999).

Chief reasons of amphibian population decline, now popular around the world, still remain largely unknown, although many causes such as habitat destruction, anthropogenic effect, and increasing ultraviolet radiation may be included (e.g., Bradford, 1991; Sherman and Morton, 1993; Blaustein, 1994; Pechmann and Wilbur, 1994). Because introduction of non-native species is also regarded as a cause of decline in native species (Hayes and Jennings, 1986; Bradford, *et al.*, 1993; Knapp and Matthews, 2000), additional biological fac-

tors such as interspecific interaction between co-inhabiting native frog species may also be responsible for some parts of amphibian decline, especially where natural environment is modified. This kind of study, however, has never been adequately made so far.

Kellner and Green (1995) suggested that the information about age of amphibians may be valuable resource in charting the rise and decline of amphibian populations in relation to climate and the impact of human activities. In this study, we studied size and age structures of *R. nigromaculata* in the breeding aggregation, in two areas with and without sympatric competing species (*R. porosa brevipoda*). Our purpose is to compare size and age parameters, such as age at first reproduction, longevity, age structure, and growth pattern, in the two populations so as to infer the effect of sympatric species.

MATERIALS AND METHODS

The study sites are rice fields located in Katata, Shiga Prefecture (35°08'N, 135°59'E, 100 m a.s.l.) and Shizuhara, Kyoto Prefecture (35°07'N, 135°52'E, 200 m a.s.l.), Japan, and have been described elsewhere (Hirai and Matsui, 1999, 2000). Briefly, the rice fields in Katata are newly modified and adjoining to city regions, while those of Shizuhara are old-fashioned and surrounded by low mountains. *Rana nigromaculata* was an explosive breeder and bred in rice fields within two weeks in a site from late April to mid May. Metamorphosis took place between late June and late July. At Katata, three *Rana* (*R. p. brevipoda*, *R. rugosa*, and *R. catesbeiana*), one hylid, and two

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rhacophorid species occurred besides *R. nigromaculata*, while at Shizuhara *R. nigromaculata* co-occurred in rice fields with one *Rana* (*R. rugosa*), one hylid, and two rhacophorid species. Of these, *R. p. brevipoda* and *R. rugosa* stayed with *R. nigromaculata* in the rice fields throughout year, and all the others utilized there only as breeding sites. *Rana catesbeiana* was found mainly in ditches and ponds nearby.

We carried out two kinds of fieldworks. First, we made collection of frogs in 1995 and 1996 as a part of stomach content analyses. The study periods were from 13 May to 13 October 1995 and from 10 May to 6 October 1996 in Katata, and from 24 April to 23 October of 1995 and from 24 April to 22 October of 1996 in Shizuhara. We captured frogs, extracted their stomach contents by forced regurgitation, measured snout-vent length (SVL), weighed body mass (BM), marked them by toe-clipping for individual identification, and released them at the point of their capture. Stomach content analyses were made following methods described in Hirai and Matsui (1999, 2001), and inter-population variations of food resource utilization were examined by comparing mean size of prey, number of food items ingested, and relative volume of food volume (mm^3) to frog body mass (g). Clipped digits were preserved in 10% formalin for skeletochronology. As a result we marked 733 individuals, of which seven recaptured specimens were utilized for skeletochronology.

Another fieldwork was conducted mainly in breeding and metamorphosing seasons of *R. nigromaculata* in 1998 and 1999. We collected breeding adults (63 males and 10 females from Katata and 72 males and 24 females from Shizuhara) in this period. We also collected newly metamorphosing or just metamorphosed froglets on 29 June and 1 and 23 July 1999 at Katata (N=42) and on 5, 6, and 22 July 1999 at Shizuhara (N=67), and juveniles on 6 August and 3 September 1999 at Katata (N=39) and on 6 August and 4 September 1999 at Shizuhara (N=55). We could easily sex adult males by the presence of secondary sexual characteristics such as vocal sacs and nuptial pads. We could also differentiate adult females from juveniles by their distinctly larger size and gravid condition. Stages of larval development were determined following Gosner (1960), and froglets of Stage 46 or older were used to compare SVL, body mass, and degree of fatness between the two populations. We calculated the degree of fatness as ratio of 10^4 body mass to SVL^3 .

We first examined various fingers from five frogs to evaluate the

appropriate bone for age study. Fingers preserved in formalin were washed in running water for 24 hr, decalcified in 5% nitric acid for 60–90 min, and washed again in running water for 24 hr. Using a freezing microtome we cross-sectioned phalanges (20–22 μm thick) and stained them with hematoxylin (Mayer's acid hemalum) for 30 min. Because the resting line was absent or incomplete in the epiphysis, we selected sections from the central region of diaphysis, and mounted in glycerin after rinsing in the tap water. We examined sections under a light microscope and counted the number of lines of arrested growth (LAGs) present in the periosteal bone. The counts of LAG numbers were checked by two of us (KW and YM). In order to specify the age of each individual frog, the time of capture in the year was taken into consideration, and in calculating ages, we set the starting point arbitrarily on 1 May when breeding was at its peak. Diameter of the innermost portions of the periosteal bone (resorption line) was measured using an ocular micrometer.

Logistic Model $L=a/(1+e^{-bt})$, where L is the SVL (mm) at time t (yr); a is the asymptotic size; c is the constant determined by putting 0 to t; and b is the instantaneous growth rate, was used to estimate the growth pattern (Misawa and Matsui, 1999). We also analyzed growth pattern for seven marked and recaptured frogs from Shizuhara.

We examined differences in fatness and age composition by Mann-Whitney U-test, while we applied Students' t-test for body size comparisons. The significance level was set at 0.05.

RESULTS

Body size and food consumption difference between populations

Because neither SVLs nor age structure differed significantly within a population between 1998 and 1999 breeding seasons ($P>0.05$), data for two years were pooled. In both Shizuhara and Katata populations, females were significantly larger in SVL and BM than males ($P<0.05$; Table 1), but degree of fatness did not differ between the sexes in Katata ($P>0.05$). Males of Shizuhara were significantly larger in the mean SVL and BM than those of Katata ($P<0.05$), but the

Table 1. Number of individuals, snout-vent length (SVL in mm), body mass (BM in g), age (year), and degree of fatness of adult *R. nigromaculata* from Katata and Shizuhara populations. Mean \pm 2SE are given. Ranges are shown in parentheses.

Population	Sex	N	SVL	BM	Age	Degree of fatness
Katata	M	63	63.6 \pm 1.96 (51.8–81.0)	22.7 \pm 2.12 (12.0–41.4)	3.27 \pm 0.3 (2.0–5.0)	0.854 \pm 0.012 (0.462–1.100)
	F	10	75.9 \pm 3.98 (68.2–85.9)	36.5 \pm 5.28 (24.2–51.5)	4.10 \pm 0.56 (3.0–5.0)	0.828 \pm 0.031 (0.633–0.992)
Shizuhara	M	72	66.7 \pm 1.22 (55.0–75.8)	24.9 \pm 1.42 (13.6–39.4)	3.42 \pm 0.24 (2.0–6.0)	0.826 \pm 0.008 (0.449–0.980)
	F	24	76.3 \pm 2.96 (60.5–90.8)	40.1 \pm 5.74 (18.8–76.0)	4.08 \pm 0.30 (3.0–6.0)	0.871 \pm 0.022 (0.653–1.091)

Table 2. Mean \pm 2SE of snout-vent length (SVL in mm), body mass (BM in g), and degree of fatness in the just metamorphosed froglets (stage 46 of Gosner, 1960) from Katata and Shizuhara populations.

Population	N	SVL*	BM*	Degree of fatness #
Katata	42	25.19 \pm 0.28	1.38 \pm 0.05	0.855 \pm 0.011
Shizuhara	67	22.73 \pm 0.25	0.90 \pm 0.04	0.751 \pm 0.011

* Significant difference by Student t-test

Significant difference by Mann-Whitney U-test

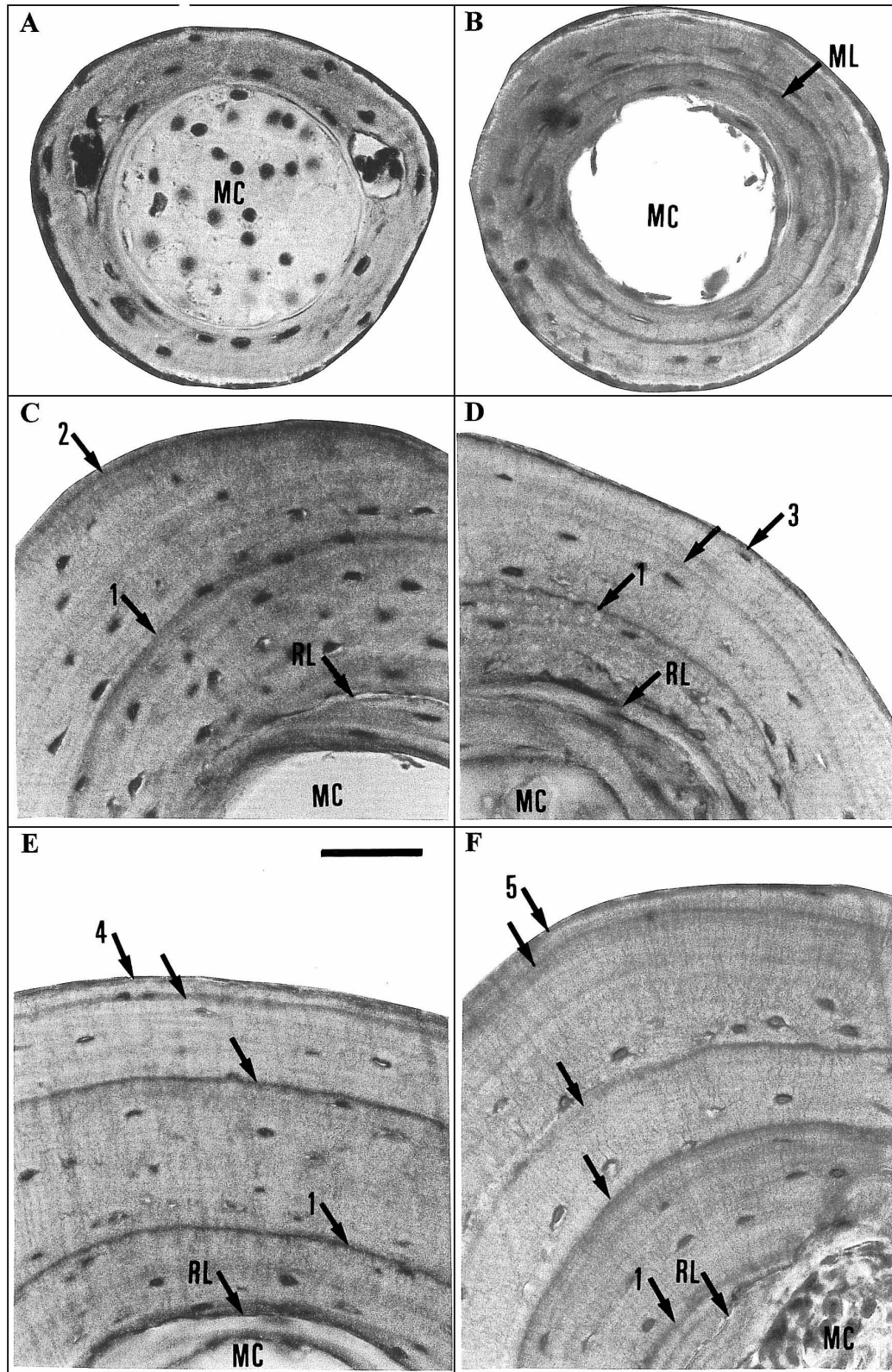


Fig. 1. Phalangeal bone cross-section of *R. nigromaculata*;

(A) metamorphosed froglet with 23.7 mm SVL collected on 29 June 1999 showing no ML or LAGs, (B) juvenile with 27.7 mm SVL collected on 4 September 1999 showing 1 ML, (C) male with 61.0 mm SVL collected on 5 May 1999 showing 2 LAGs, (D) female with 70.5 mm SVL collected on 24 April 1998 showing 3 LAGs, (E) female with 76.1 mm SVL collected on 6 May 1999 showing 4 LAGs, (F) male with 70.1 mm SVL collected on 23 April 1999 showing 4 LAG and one outer edge of bone (age class 5). MC = marrow cavity; ML = metamorphosed line; RL = resorption line; Arrows = LAGs; Scale bar = 50 μ m.

populations did not differ in BM. In females, no size parameters differed between the populations.

As shown in Table 2, just metamorphosed froglets from Katata were significantly larger in SVL, heavier in body mass, and fattier than those from Shizuhara ($P < 0.05$).

There is a variation in food parameters between the two populations. Although the number of prey items (6.9 ± 5.8 in Katata, $N=304$; 7.7 ± 7.1 in Shizuhara, $N=478$; $P > 0.05$) did not differ, volume of food relative to body mass was significantly smaller in Katata ($18.5 \pm 23.6 \text{ mm}^3/\text{g}$, $N=304$) than in Shizuhara ($39.2 \pm 40.6 \text{ mm}^3/\text{g}$, $N=478$; $P < 0.01$).

General result of skeletochronology

Among various phalanges we examined, we found the third finger to be most suitable for skeletochronology because of its round shape and minimum degree of resorption. In addition to these, most cross-sections of this finger retained a metamorphosed line (ML; Fig. 1) that is useful as a marker for counting LAGs. Froglets just after metamorphosis had neither ML nor LAG. In most juveniles collected long after metamorphosis, however, MLs as distinct as LAGs were observed. These MLs and LAGs were partly destroyed by the resorption process in some still larger individuals (Fig. 1). This was confirmed by comparing diameters of resorption lines (innermost diameter of periosteal bone) among frogs of different age classes. In this case, however, only some parts of periosteal bone were found destroyed and the process did not strongly affect the number of LAGs.

The data from recaptured frogs proved that one LAG increased per one year. The formation of a new line occurred in May. We collected breeding adults just after emergence from hibernation, when the formation of the newest ring has not yet completed. We, thus, included an incomplete line at the outer edge of periosteal bone in counting LAGs for these individuals.

Age at first reproduction and longevity

The age of breeding individuals was estimated to be from

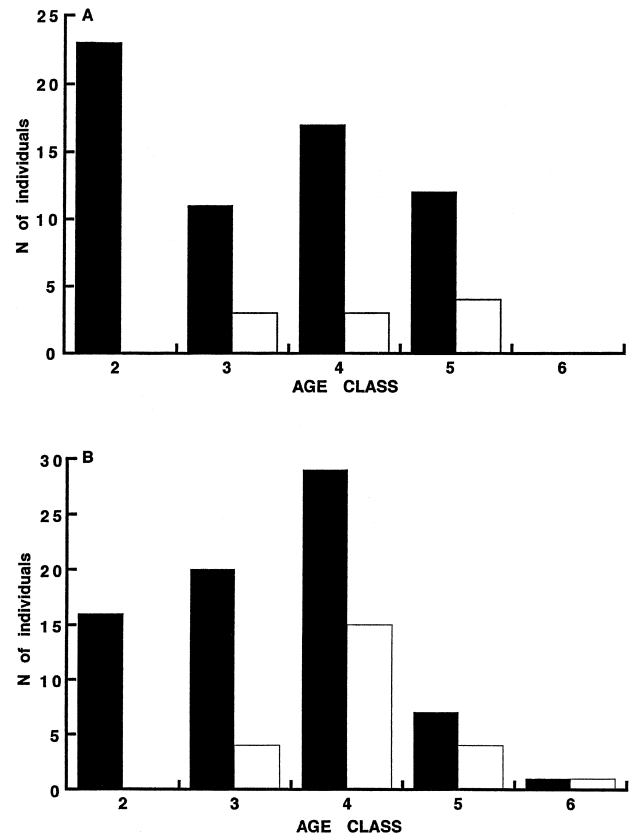


Fig. 2. Age composition distribution of males (closed bars) and females (open bars) from (A) Katata and (B) Shizuhara populations.

2 to 5 yrs in males and 3 to 5 yrs in females in Katata. In Shizuhara, ages varied from 2 to 6 yrs in males and 3 to 6 yrs in females (Table 1). Therefore, males were estimated to start breeding at the age of two years and females at three in both populations. Similarly, the longevity was estimated to be at least five and six yrs at Katata and Shizuhara, respectively.

In both populations breeding males (3.27 yrs in Katata and 3.42 yrs in Shizuhara) were significantly younger on an

Table 3. Mean \pm 2SE of snout-vent length (SVL in mm) and body mass (BM in g) of the frogs in each age class of Katata and Shizuhara populations. Ranges are shown in parentheses.

Population	Sex	Age class (yr)	N	SVL	BM
Katata	M	2	23	56.7 \pm 1.14 (51.8–61.0)	15.6 \pm 1.06 (12.0–19.8)
		3	11	61.3 \pm 2.74 (56.0–69.2)	21.3 \pm 3.12 (15.8–31.0)
		4	17	66.1 \pm 2.22 (59.1–74.5)	24.4 \pm 2.58 (16.8–34.2)
		5	12	75.3 \pm 1.71 (70.1–81.0)	35.3 \pm 3.50 (17.6–41.4)
		6	0		
Katata	F	3	3	69.2 \pm 1.38 (68.2–70.5)	27.4 \pm 4.72 (24.2–32.0)
		4	3	74.8 \pm 0.60 (74.5–75.4)	37.2 \pm 5.25 (32.2–41.0)
		5	4	81.8 \pm 4.94 (74.8–85.9)	42.9 \pm 6.86 (36.6–51.5)
		6	0		
		6	0		
Shizuhara	M	2	16	59.4 \pm 1.36 (55.0–65.0)	17.7 \pm 1.50 (13.6–25.0)
		3	19	66.1 \pm 1.20 (60.3–71.9)	24.1 \pm 1.64 (17.6–31.2)
		4	29	70.0 \pm 1.24 (65.5–75.8)	28.7 \pm 1.70 (22.0–39.4)
		5	7	71.2 \pm 1.74 (68.5–75.5)	27.8 \pm 5.18 (15.4–36.4)
		6	1	68.5	27.2
		6	0		
Shizuhara	F	3	4	66.4 \pm 4.58 (60.5–70.0)	24.9 \pm 4.86 (18.6–30.0)
		4	15	77.4 \pm 2.42 (67.8–84.5)	40.9 \pm 5.04 (25.4–55.0)
		5	4	78.4 \pm 7.88 (72.0–89.2)	43.6 \pm 17.0 (30.4–66.5)
		6	1	90.8	76.0

average than females (4.10 yrs in Katata and 4.08 yrs in Shizuhara; $P<0.05$), but in the mean age, each sex did not differ between the two populations ($P>0.05$).

Age structure

As shown in Fig. 2, males of the age class 2 in the Katata population and 4 in the Shizuhara population were most abundant at the time of breeding (36.5% and 40.3%, respectively, of the total number). The median value was also different between Katata (3 yrs old) and Shizuhara (4 yrs old). These

results suggested a predominance of older males in Shizuhara than in Katata (Fig. 2), but the age structure of breeding males did not differ between the two populations ($P>0.05$). In females, small sample number prohibited exact comparisons, but the age class 4 predominated (62.0% of the total number) in Shizuhara.

Growth pattern

Between the males of two populations, SVL and BM differed in each of 2–5 year classes significantly ($P<0.05$), with the exception of BM in age class 3. In all cases, Shizuhara population was significantly larger than Katata. By contrast, females in each age class did not differ interpopulationally, probably because of small sample size. Sexual size dimorphism in SVL was evident in each age class and females were invariably larger than males in both populations (Table 3). From these results, growth pattern was estimated by separating sexes and populations.

Growth curves estimated by a Logistic Model and the growth parameters calculated are shown in Fig. 3 and Table 4, respectively. Asymptotic size was different between the sexes in each population, although it did not differ in the same sex between the two populations. The instantaneous growth rate in males, however, was larger in Shizuhara than in Katata. In females, the instantaneous growth rate did not differ

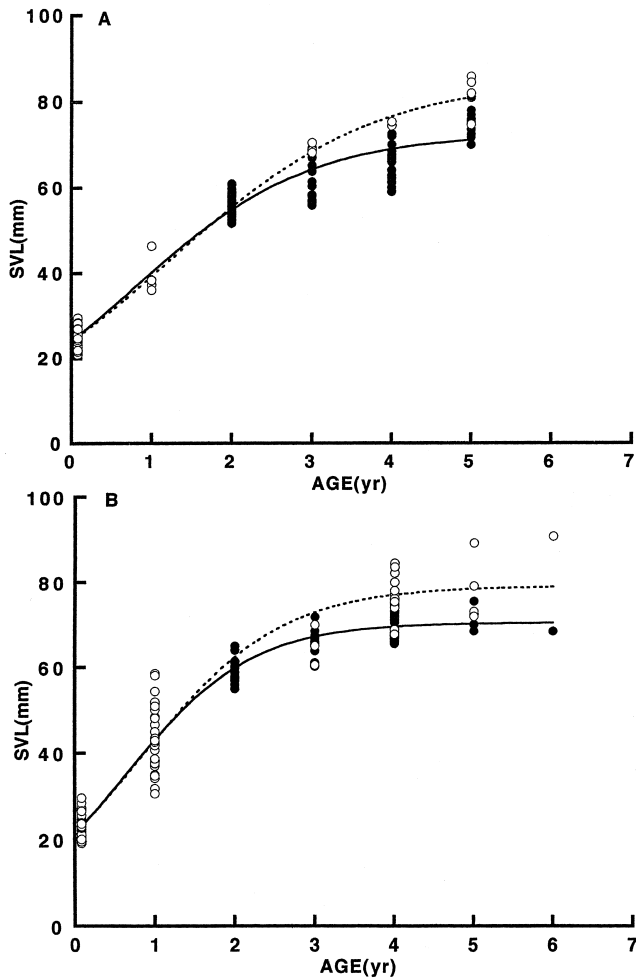


Fig. 3. Growth curves estimated by Logistic Model of males (closed circles) and females (open circles) from (A) Katata and (B) Shizuhara populations.

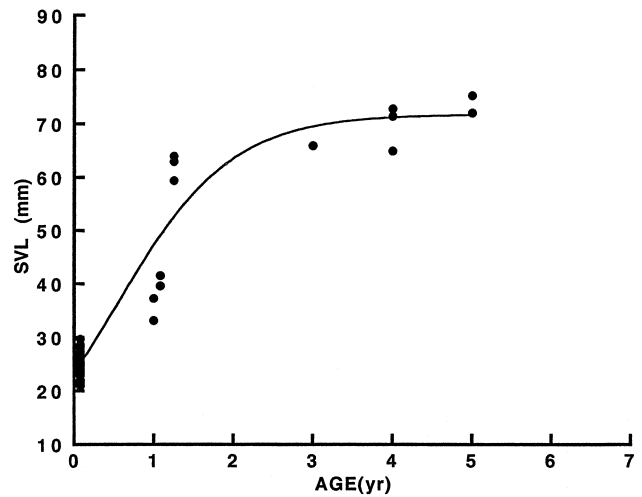


Fig. 4. Growth curves estimated by logistic model of recaptured frogs from Shizuhara population.

Table 4. Growth parameters (mean \pm 2SE) estimated by a Logistic Model for skeletochronological data of Katata and Shizuhara populations and recaptured data for Shizuhara population.

	Skeletochronological data				Recaptured data
	Katata		Shizuhara		Shizuhara
Sex	M	F	M	F	M
N	63	10	72	24	7
Asymptotic size	72.53 \pm 2.011	85.25 \pm 3.59	70.48 \pm 1.328	79.08 \pm 2.53	71.74 \pm 3.58
Instantaneous growth rate	0.9184 \pm 0.0829	0.7786 \pm 0.0858	1.2802 \pm 0.0998	1.1513 \pm 0.1284	1.3954 \pm 0.2164

between the two populations, possibly affected by their small sample size.

For a small number of recaptured frogs from Shizuhara, including individuals that were recaptured within a year (juvenile males, $N=4$) and over a year (adult males, $N=3$), Logistic Model was also applied to determine the growth pattern (Fig. 4). The growth pattern thus obtained demonstrated that neither the asymptotic size nor instantaneous growth rate differ from the values obtained from skeletochronological data (Table 4).

DISCUSSION

Skeletochronology

In *R. nigromaculata*, no growth line was formed in long bones at the time of metamorphosis, but a ML and LAGs were formed subsequently. Although these lines were partly destroyed by the resorption process in older individuals, such a process did not affect LAG counts. Formation of one LAG per one year was confirmed from recaptured frogs and a new line seemed to become evident after the breeding season in May.

As already indicated by previous studies on age determination (e.g., Halliday and Verrell, 1988; Paton *et al.*, 1991), LAGs of temperate amphibian species are formed in correspondence with annual environmental changes such as temperature fluctuation, and this was the case in *R. nigromaculata*. By comparing mark-recapture and skeletochronological data in a lizard, Mouden, *et al.* (1999) indicated that the skeletochronological data underestimate the asymptotic size of the lizard. By contrast, the results of our study showed that mark-recapture and skeletochronological data are not different with relation to growth parameters and confirmed that the method of skeletochronology is reliable and suitable for studying growth at least in *R. nigromaculata*.

Interpopulation comparison

In both populations of *R. nigromaculata*, males started breeding at 2 and females at 3 yrs of age, and thus breeding males were younger on an average than females. These values of first breeding may be viewed as moderate when compared with published results of the other anuran species (2 yrs in males and 2-3 yrs in females: e.g., Tinsley and Tocque, 1995; Kuzmin and Ischenko, 1997; Sullivan and Fernandez, 1999). The fact that males tend to be younger than females has been reported in various anuran species (e.g., Cherry and Francillon-Vieillot, 1992; Kusano *et al.*, 1995a; Ryser, 1996; Marquez *et al.*, 1997). Males would breed as soon as they mature reproductively whereas females would not attempt to breed until reaching a critical size (Cherry and Francillon-Vieillot, 1992). This delayed maturity in females must have resulted from the individual investment of its energy resource for production of eggs (Guarino *et al.*, 1998).

There are several marked differences in the two populations of *R. nigromaculata*. Froglets just after metamorphosis were larger, heavier, and more fatty in Katata than in Shizu-

hara. Many factors, such as temperature, density of conspecifics, and duration of permanent water, are considered to affect the size at metamorphosis (e.g., Amezcuita and Luddecke, 1999), and our results indicate the presence of such environmental differences between the two populations. In instantaneous growth rate and mean SVL in males, and in volume of food relative to body mass, Katata population had smaller values than in Shizuhara, although they did not differ in asymptotic size. Because frogs from Shizuhara metamorphosed at a smaller size, they could reach an asymptotic size similar to the frogs from Katata by a higher growth rate, probably owing to more prey that are available.

The modal age of Katata males (2 yrs), in contrast to 4 yrs in Shizuhara, indicated a predominance of younger breeding males in this population, although the mean age and age structure did not differ between the two populations. The occurrence of larger metamorphs but younger adult males in Katata than in Shizuhara partly agrees with Berven's (1990) prediction that larger juveniles would attain sexual maturity at younger age, and again, variation in age structures of two *R. nigromaculata* populations seems to have been affected by local variations in biotic and abiotic factors that affect growth and survivorship (Caetano and Leclair, 1996; Ryser, 1996).

Further, the maximum life span seemed to differ between the two populations (at least 5 yrs in Katata v.s. 6 yrs in Shizuhara), and this differential longevity might be also a result of environmental effects. Because longevity of *R. nigromaculata* is short compared with some other anuran species (8-13 yrs: e.g., Kuzmin and Ischenko, 1997), even one year difference may be not meaningless.

Interspecific competition

The absence of competition is expected to increase survival, growth, and fecundity (Berven, 1990), and our results of *R. nigromaculata* seem to agree with such an assumption, because the Katata population is syntopic with *R. porosa brevipoda*, which is absent in Shizuhara. *Rana p. brevipoda* and *R. nigromaculata* are phylogenetically close to each other and generally occupy a similar ecological niche (Maeda and Matsui, 1999). Indeed, higher growth rate, higher rate of food consumption, a larger size in most age classes, and greater number of older breeding individuals were observed in Shizuhara (without syntopic species with similar niche) than in Katata population (with syntopic species).

Skeletochronology in relation to conservation

We thus consider that the differences found in age and size properties between the two populations of *R. nigromaculata* might have resulted from some biological factors such as food availability and interspecific competition that are different between the populations. In this way, our study of skeletochronology offers some insight into popular topics of amphibian declining problems. Interspecific competition in Katata may have been strengthened through artificial modification of rice fields where frogs inhabit. Further ecological studies with relation to habitat modification by human would surely

contribute for conservation of *R. nigromaculata* and many other frog species inhabiting rice fields of Japan, and skeleto-chronology would occupy an important part of such studies.

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